ORGANIC LETTERS

2001
Vol. 3, No. 7
987−990

A 2′-Acylamido Cap That Increases the Stability of Oligonucleotide Duplexes

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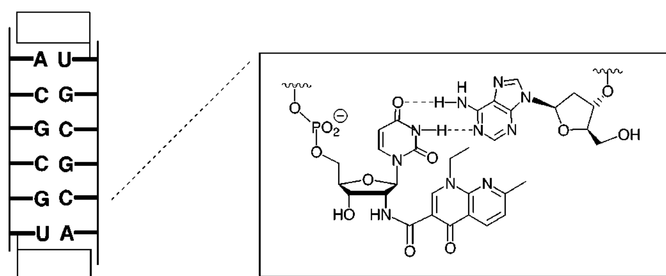
Received December 11, 2000

ABSTRACT



Reported here is the synthesis of oligonucleotides containing a 2′-acylamido-2′-deoxyuridine residue at their 3′-terminus. Compared to control sequences bearing a thymidine residue, the quinolone-capped duplexes give higher UV melting points. In the case of $(5'\text{-ACGCGU-NA-}2')_2$, where NA denotes a nalidixic acid residue, the melting point increase is up to 22 °C over that of $(ACGCGT)_2$.

The hybridization of oligonucleotides to complementary strands is a key molecular recognition event for biomedical applications such as the measurement of gene expression with DNA chips,[1] the inhibition of gene expression with antisense agents,[2] or the cleavage of target strands with designed ribozymes.[3] While the Watson−Crick base pairing rules apply to oligonucleotides of almost any sequence, the stability of the resulting duplexes varies greatly with the sequence. In particular, T:A and A:T base pairs destabilize duplexes, both in the interior and at the termini of the double helix.[4] This makes it desirable to develop modified adenosine and thymidine derivatives that form base pairs of a stability similar to that of C:G and G:C pairings, thus allowing for the generation of oligonucleotide probes whose affinity for a target strand is independent of the G/C content.

We have previously reported modifications for the 5′- and 3′-termini of oligonucleotides with 5′-terminal thymidine residues.[5−7] Some of these were found to increase the affinity of the oligonucleotides for target strands.[8] We are therefore referring to these modifications as "molecular caps".[9] Molecular modeling results obtained during a search for new cap structures suggested that an acyl substituent at the 2′-position of a terminal ribonucleoside in a B-form duplex of

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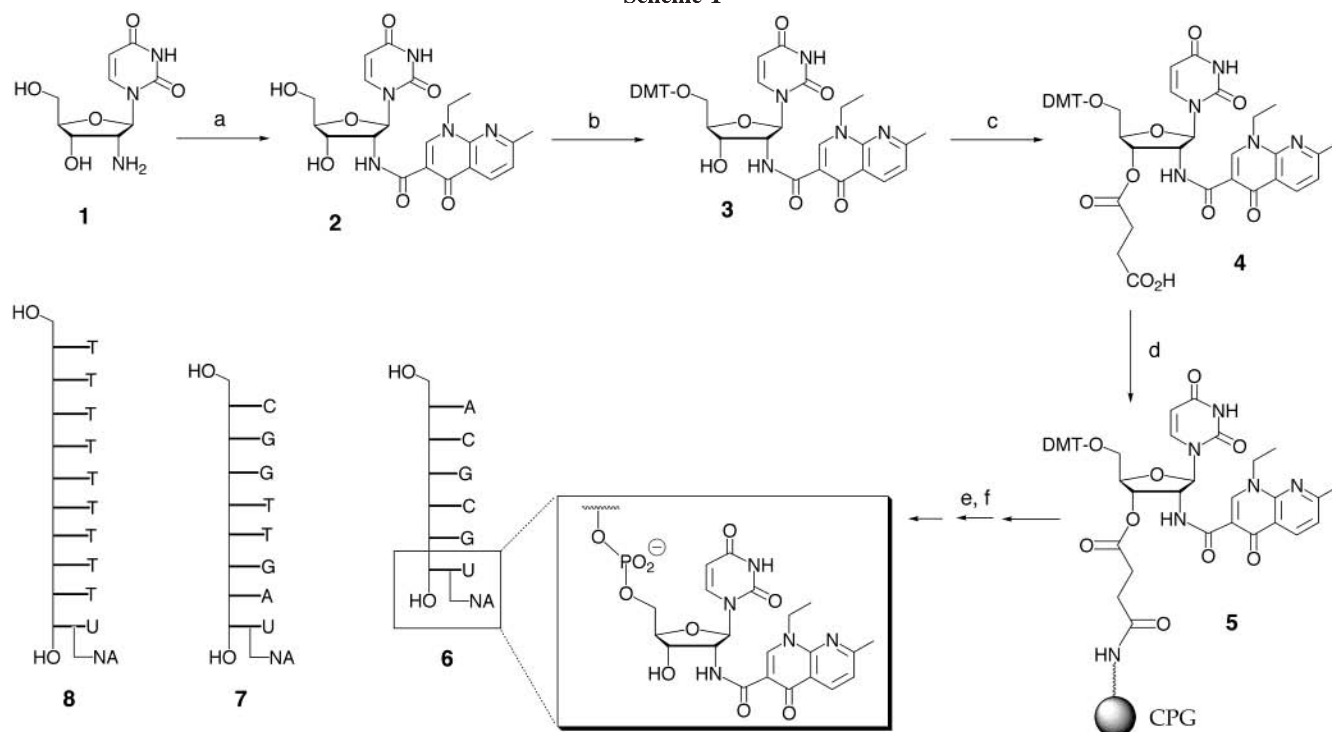
10.1021/ol0003782 CCC: $20.00 © 2001 American Chemical Society
Published on Web 03/09/2001

Scheme 1[a]



[a] (a) Nalidixic acid, HBTU, DIEA, DMF; (b) DMT-Cl, DMAP, TEA, pyridine; (c) succinic anhydride, DMAP, pyridine; (d) LCAA-cpg, HBTU, HOBT, DIEA, DMF; (e) DNA synthesis via the phosphoramidite protcol; (f) NH_4OH.

an oligonucleotide containing otherwise only 2′-deoxynucleotides could stack on the terminal base pair and thus potentially stabilize the duplex against denaturation.[10] 2′-Acylamidonucleotides have previously been employed in oligoribonucleotides and oligodeoxyribonucleotides to introduce new functionalities for cross-linking,[11] to induce chemical cleavage of DNA,[12] to render them fluorescent,[13] and to obtain models for reporter group-bearing strands.[14] The 2′-acylated residues include adenosine derivatives with 2′-acylamido substituents.[15] Unacylated 2′-amino-2′-deoxyuridine residues have been used to modify hammerhead ribozymes[16,17] and to provide insight into the mechanism of RNA hydrolysis.[18]

Reported here is the synthesis of oligodeoxynucleotides whose 3′-terminal nucleotide is a 2′-amino-2′-deoxyuridine residue acylated with a nalidixic acid residue. A structurally similar quinolone (oxolinic acid) has recently been found to stabilize DNA duplexes when appended to the 5′-terminus of one of the strands.[8c] The nalidixic acid-bearing 3′-terminal residue was found to stabilize DNA duplexes, with melting point increases of up to 22 °C over control duplexes. At the same time, the 2′-acylated residue increased the hyperchromicity associated with duplex dissociation for two of the three sequences tested.

The synthesis of the 2′-capped oligonucleotides started from 2′-amino-2′-deoxyuridine[19−21] (**1**). Coupling with nalidixic acid gave acylated nucleoside **2** in 72−91% yield (Scheme 1).[22] Protection of the 5′-hydroxyl group and succinylation of the 3′-position under standard conditions[23] proceeded uneventfully, though in moderate yield (59% for **3** and 62% for **4**).[24] Coupling of **4** to long chain alkylamine controlled pore glass (LCAA-cpg) under peptide coupling conditions[25] gave nucleoside-bearing support **5** with a loading of 24 μmol/g, as determined by dimethoxytrityl cation release. Using this support and standard phosphoramidite chemistry, self-complementary hexamer **6** was obtained[26] in high yield (71% according to the integration of the HPLC chromatogram of the crude), and its structure was confirmed by MALDI-TOF mass spectrometry (see Supporting Information). Similarly, mixed sequence, non self-complementary

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octamer **7** and all-pyrimidine decamer **8** were synthesized (Scheme 1).[27] Standard automated oligonucleotide syntheses furnished control sequences ACGCGT (**9**), ACGCGU (**10**), CGGTTGAT (**11**), and T_{10} (**12**) as well as target strands GCCAACTA (**13**) and A_{10} (**14**). The 3′-terminal residue in **10** is a ribonucleoside, incorporated by starting the synthesis with uridine-loaded cpg, whereas otherwise all sequences are composed of 2′-deoxyresidues.

Solutions of duplexes containing the 2′-acylamidouridine residue were subjected to UV melting analysis under two different buffer conditions (Table 1). In all cases the 2′-appendage increased the melting points over those of the control compounds. This effect was strongest for the hexamer duplex, with increases ranging from 13.3 °C at low salt in ammonium acetate buffer to 22 °C at high salt in phosphate-buffered NaCl. The increase in stability was accompanied by an increase in hyperchromicity, most pronounced at 1 M NaCl and 10 mM phosphate buffer (Figure 1 and Table 1).

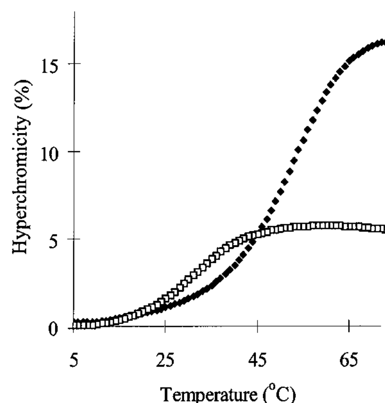

Figure 1. UV melting curves of 2′-capped $(ACGCGU\text{-}NA)_2$ (**6**, filled diamonds) and control duplex $(ACGCGT)_2$ (**9**, open squares) at 260 nm detection wavelength, in a solution containing 1 M NaCl and 10 mM phosphate buffer, pH 7.0.

The duplexes of non-self-complementary strands **7** and **8** with **13** and **14**, respectively, also showed melting point increases over their controls, though these were less dramatic than for the shorter sequence. For the all-pyrimidine duplex (**8**:**14**), melting was associated with moderately increased hyperchromicity, whereas for the mixed octamer duplex, whose penultimate residue from the 3′-terminus is an adenosine (**7**:**13**), it was not. Again, the duplex stabilizing effect of the 2′-cap was found to be strongest at high salt concentration.

When non-self-complementary octamer **7** was hybridized to target undecamer **15**, whose 5′-terminal nucleotides could either interfere with the capping effect or enhance it by stacking with the cap, a similar increase in melting point over the control duplex was found as in the system without the "dangling" residues (**7**:**13**, **11**:**13**, Table 1). This indicates that the 2′-capped strands may be good hybridization probes for targeting specific regions in long DNA sequences.

These results are noteworthy in light of previous reports indicating that 2′-acetamido-2′-deoxyuridine residues in the interior of oligonucleotides lower the melting points of their duplexes with target strands.[14] The authors proposed that the decrease in duplex stability was due to steric interference of the 2′-substituents with duplex formation. Oligonucleotides containing 2′-amino-2′-deoxycytidine residues prepared by

(22) **Compound 2**. Nalidixic acid (20 mg, 86 μmol) and HBTU (30.9 mg, 82 μmol, 0.95 equiv) were dried at 0.1 Torr for 1 h, dissolved in DMF (0.6 mL), and DIEA (0.034 mL, 198 μmol, 2.3 equiv) was added. After 10 min, the solution was added to 2′-amino-2′-deoxyuridine (**1**) (15.7 mg, 64.5 μmol, 0.75 equiv) that had been coevaporated with pyridine and dried under vacuum. After 3 h, the reaction mixture was precipitated with 10 mL of cold diethyl ether. The precipitate was dissolved in acetone/methanol (6:4) and chromatographed on silica with acetone. Yield 27 mg (59 μmol, (91%). A reaction on a 0.6 mmol scale gave 72% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 1.36 (t, J = 7.0 Hz, 3H); 2.66 (s, 3H); 3.64 (br s, 2H); 4.02 (br s, 1H); 4.17 (t, J = 5.0 Hz, 1H); 4.54 (q, J = 8.4 Hz, 2H); 4.68 (q, J = 5.6 Hz, 1H); 5.26 (s, 1H); 5.73 (d, J = 8.0 Hz, 1H); 5.99 (d, J = 8.5 Hz, 1H); 6.10 (d, J = 5.3 Hz, 1H); 7.48 (d, J = 8.2 Hz, 1H); 8.01 (d, J = 8.1 Hz, 1H); 8.55 (d, J = 8.1 Hz, 1H); 8.97 (s, 1H); 10.35 (d, J = 8.2 Hz, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 14.9, 24.7, 45.9, 54.4, 61.6, 70.9, 86.2, 86.9, 101.9, 111.4, 119.6, 121.4, 135.8, 140.8, 148.0, 148.2, 150.7, 163.0, 163.0, 163.9, 175.5. UV (H_2O, qual.) λ_{max} 261 (100%); 328 (30%). MALDI-TOF MS (positive, linear mode) m/z 480 $[M + Na]^+$; 458 $[M + H]^+$.

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(24) **Compound 3**. Acylated nucleoside **2** (150 mg, 0.33 mmol) was coevaporated twice with pyridine, dried at 0.1 Torr, treated with DMAP (1.3 mg, 10.6 μmol) and DMT-Cl (153 mg, 0.45 mmol), followed by additional drying and addition of pyridine (2.2 mL) and TEA (0.06 mL, 0.43 mmol) under argon. After stirring for 3 h, additional DMT-Cl (76 mg, 0.224 mmol) and TEA (0.03 mL, 0.215 mmol) were added, followed by stirring for an additional 2 h. Methanol (0.6 mL) was added, and the solution was evaporated and dried. The residue was taken up in CH_2Cl_2 and washed with bicarbonate solution and brine. After drying over Na_2SO_4, the crude was chromatographed (silica, CH_2Cl_2/TEA 99:1, methanol step gradient 0−3%) to yield 147 mg (0.194 mmol, 59%) of the title compound. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 1.37 (t, J = 6.9 Hz, 3H); 2.66 (s, 3H); 3.37 (m, 1H); 3.40 (m, 1H); 3.74 (s, 6H); 4.11 (br s, 1H); 4.22 (s, J = 5.2 Hz, 1H); 4.55 (q, J = 7.2 Hz, 2H); 4.85 (q, J = 6.1 Hz, 1H); 5.45 (d, J = 8.1 Hz, 1H); 5.98 (d, J = 8.2 Hz, 1H); 6.16 (d, 1H); 6.92 (m, 4H); 7.30 (m, 5H); 7.34 (m, 2H); 7.41 (m, 2H); 7.48 (d, J = 8.2 Hz, 1H); 7.78 (d, J = 8.1 Hz, 1H); 8.55 (d, J = 8.1 Hz, 1H); 9.03 (s, 1H); 10.38 (d, J = 7.9 Hz, 1H); 11.3 (br s, 1H). FABMS m/z 782 (M + Na$^+$, 4%); 760 (M + H$^+$, 0.3%); 303 (DMTr$^+$, 100%). **Compound 4**. DMT-protected **3** (45 mg, 59 μmol) was coevaporated with pyridine, dried at 0.1 Torr, treated with DMAP (3.7 mg, 30 μmol), dried again, and treated with pyridine (0.12 mL) under argon. Succinic anhydride (4.8 mg, 48 μmol) was added in three portions over 30 min under stirring. After 16 h, the reaction mixture was coevaporated twice with toluene, the residue was dissolved in CH_2Cl_2, and the resulting solution was washed twice each with ice-cold citric acid solution (3.5%) and ice-cold water. The organic phase was dried over Na_2SO_4 and evaporated, and the residue was dissolved in CH_2Cl_2 (0.2 mL), precipitated into stirred cold hexane (5 mL), and isolated by centrifugation and drying at 0.1 Torr. Yield 31.8 mg (37 μmol, 62%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 1.37 (t, J = 6.9 Hz, 3H); 2.57 (m, 2H); 2.67 (s, 3H); 2.76 (m, 2H); 3.34 (m, 1H); 3.44 (m, 1H); 3.75 (s, 6H); 4.24 (br s, 1H); 4.56 (q, J = 7.2 Hz, 2H); 5.19 (pseudo q, J = 6.3 Hz, 1H); 5.31 (d, J = 6.5 Hz, 1H); 5.51 (d, J = 8.1 Hz, 1H); 6.01 (d, J = 8.6 Hz, 1H); 6.92 (m, 4H); 7.26 (m, 5H); 7.34 (m, 2H); 7.42 (m, 2H); 7.50 (d, J = 8.2 Hz, 1H); 7.77 (d, J = 8.2 Hz, 1H); 8.56 (d, J = 8.1 Hz, 1H); 9.04 (s, 1H), 10.48 (d, J = 8.6 Hz, 1H); 11.41 (br s, 1H). FAB MS m/z 882 (M + Na$^+$, 1%); 303 (DMTr$^+$, 2%).

(25) **Compound 5**. Succinylated nucleoside **4** (6.7 mg, 7.9 μmol) was dried at 0.1 Torr, treated with HBTU (2.7 mg, 7.1 μmol, 0.9 equiv) and HOBT (1.2 mg, 7.9 μmol, 1 equiv), followed by additional drying and addition of DMF (0.1 mL) and DIEA (3.2 μL). Then, LCAA-cpg (12 mg, 1.12 μmol of amino groups) was added and the mixture vortexed every 15 min. After 2.5 h, the supernatant was aspired and the cpg washed three times with DMF and four times with CH_3CN, followed by drying at 0.1 Torr.

(26) **ACGCGU-NA** (**6**): yield 71%. HPLC: CH_3CN gradient 0% for 5 min, to 25% in 40 min, elution at 41.3 min. MALDI-TOF MS for $C_{63}H_{84}N_{35}O_{36}P_5$ $[M - H]^-$: calcd 2007.8, found 2006.0.

(27) **CGGTTGAU-NA** (**7**): yield 85%. HPLC: CH_3CN gradient 0% for 10 min, to 30% in 30 min, elution at 29.44 min. MALDI-TOF MS for $C_{90}H_{110}N_{32}O_{50}P_7$ $[M - H]^-$: calcd 2656.9, found 2655.7. **TTTTTTTTTU-NA** (**8**): yield 95%. HPLC: CH_3CN gradient 0% for 10 min, to 30% in 30 min, elution at 26.95 min. MALDI-TOF MS for $C_{111}H_{110}N_{32}O_{50}P_7$ $[M - H]^-$: calcd 3196.2, found 3193.8.

Table 1. UV Melting Points and Hyperchromicities of Duplexes

duplex[a]	T_m (°C)[b]	ΔT_m (°C)[c]	hyperchromicity (%)[b]
10 mM NH_4OAc			
$(ACGCGT)_2$ (**9**)$_2$	22.2 ± 0.5		8.6 ± 0.3
$(ACGCGU)_2$ (**10**)$_2$	22.2 ± 0.4	0.0	9.4 ± 0.2
$(ACGCGU\text{-}NA)_2$ (**6**)$_2$	35.5 ± 0.5	+13.3	15.4 ± 0.6
CGGTTGAT:ATCAACCG (**11**:**13**)	<15		
CGGTTGAU-NA:ATCAACCG (**7**:**13**)	<15		
T_{10}:A_{10} (**12**:**14**)	<15		
T_9U-NA:A_{10} (**8**:**14**)	<15		
150 mM NH_4OAc			
$(ACGCGT)_2$ (**9**)$_2$	33.6 ± 1.0		9.0 ± 0.3
$(ACGCGU)_2$ (**10**)$_2$	33.6 ± 1.0	0.0	9.4 ± 0.5
$(ACGCGU\text{-}NA)_2$ (**6**)$_2$	48.0 ± 0.9	+14.4	16.5 ± 0.4
CGGTTGAT:GCCAACTA (**11**:**13**)	30.4 ± 0.4		20.9 ± 1.0
CGGTTGAU-NA:GCCAACTA (**7**:**13**)	34.2 ± 0.3	+3.8	18.8 ± 0.3
T_{10}:A_{10} (**12**:**14**)	25.7 ± 0.4		25.4 ± 0.3
T_9U-NA:A_{10} (**8**:**14**)	31.0 ± 0.3	+5.3	30.2 ± 0.3
CGGTTGAT:ACTATCAACCG (**11**:**15**)	34.7 ±0.7		18.2 ± 0.4
CGGTTGAU-NA:ACTATCAACCG (**7**:**15**)	40.1 ± 0.7	+5.4	14.9 ± 0.7
1000 mM NH_4OAc			
$(ACGCGT)_2$ (**9**)$_2$	33.9 ± 0.7		8.5 ± 0.2
$(ACGCGU)_2$ (**10**)$_2$	34.1 ± 0.8	+0.2	9.0 ± 0.2
$(ACGCGU\text{-}NA)_2$ (**6**)$_2$	50.5 ± 2.0	+16.6	13.7 ± 1.9
CGGTTGAT:ATCAACCG (**11**:**13**)	35.1 ± 0.9		17.4 ± 0.8
CGGTTGAU-NA:ATCAACCG (**7**:**13**)	40.2 ± 0.8	+5.1	13.3 ± 1.0
T_{10}:A_{10} (**12**:**14**)	36.0 ± 0.5		26.3 ± 0.4
T_9U-NA:A_{10} (**8**:**14**)	42.3 ± 0.4	+6.3	29.9 ± 0.6
CGGTTGAT:ACTATCAACCG (**11**:**15**)	40.2 ± 0.7		16.8 ± 1.3
CGGTTGAU-NA:ACTATCAACCG (**7**:**15**)	44.8 ± 0.6	+4.6	15.6 ± 0.3
10 mM phosphate buffer			
$(ACGCGT)_2$ (**9**)$_2$	20.6[d]		6.3[d]
$(ACGCGU\text{-}NA)_2$ (**6**)$_2$	35.2[d]	+14.6	16.6[d]
150 mM NaCl, 10 mM phosphate buffer			
$(ACGCGT)_2$ (**9**)$_2$	31.7 ± 0.7		7.9 ± 2.0
$(ACGCGU\text{-}NA)_2$ (**6**)$_2$	52.6 ± 1.3	+20.9	17.5 ± 1.7
1000 mM NaCl, 10 mM phosphate buffer			
$(ACGCGT)_2$ (**9**)$_2$	31.1 ± 0.3		6.6 ± 1.4
$(ACGCGU\text{-}NA)_2$ (**6**)$_2$	53.1 ± 0.3	+22.0	16.7 ± 0.7

[a] Sequences are given from 5′- to 3′-terminus. [b] Average of four melting points ± SD at 3.5 ±0.6 μM (**6**, **9**, **10**), 1.4 ±0.1 μM (**7**, **11**, **13**), or 1.1 ±0.15 μM (**8**, **12**, **14**) strand concentration. [c] Melting point difference to control strand. [d] Average of two melting curves.

another group, on the other hand, were also found to destabilize duplexes, both at neutral pH and at pH 5.0, even though they are not much more sterically demanding than hydroxyl groups.[28] Steric interference is probably absent when the acylamido substituent is at the 3′-terminus of the oligonucleotide forming a duplex, as seen in our studies. One case is known in which 2′-acylated 2′-deoxyuridine residues had a stabilizing effect. These residues were placed at the 5′-terminus of oligonucleotides.[13] The increase in melting point was ≤3 °C, however, possibly because the bulky substituents employed disrupt the 5′-terminal base pair.

Exploratory NMR work on the duplex of **6** shows sharp signals, suggesting that it will be possible to solve the three-dimensional structure of this 2′-capped double helix. Since the duplex-stabilizing effect of the nalidixic acid residues increases with the salt concentration (Table 1), even though the quinolone should be either uncharged or protonated at its B-ring imino nitrogen, it is reasonable to assume that the appended quinolone does not disrupt the terminal base pair. Therefore, there is hope that high fidelity hybridization probes[8d] whose otherwise T-weakened terminal positions have been enforced with caps at the 5′- and 3′-terminus may be constructed in the foreseeable future.

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Acknowledgment. The authors thank Charles Tetzlaff for acquiring MALDI spectra and for help with computer issues. This work was supported by the NSF (Grant CHE-9984142) and the Deutsche Forschungsgemeinschaft (Grant . RI 1063/1). The NMR facility at the Chemistry Department at Tufts University was supported by NSF Grant CHE-9723772.

Supporting Information Available: ^{1}H NMR spectra of **2**, **3**, and **4** and MALDI-TOF mass spectra of **6**, **7**, and **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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